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Residue Leu^{940} Has a Crucial Role in the Linkage and Reaction Specificity of the Glucansucrase GTF180 of the Probiotic Bacterium *Lactobacillus reuteri* 180*

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Background: Glucansucrases of lactic acid bacteria catalyze the synthesis of a variety of α-glucans from sucrose.
Results: Mutations of Leu^{940} in domain B of GTF180 significantly altered its linkage and reaction specificity.
Conclusion: Residue Leu^{940} of GTF180 is a critical structural determinant for linkage and reaction specificity.
Significance: This study provides novel insights into the structural basis of glucansucrases to facilitate the production of tailor-made α-glucans.

Highly conserved glycoside hydrolase family 70 glucansucrases are able to catalyze the synthesis of α-glucans with different structure from sucrose. The structural determinants of glucansucrase specificity have remained unclear. Residue Leu^{940} in domain B of GTF180, the glucansucrase of the probiotic bacterium *Lactobacillus reuteri* 180, was shown to vary in different glucansucrases and is close to the +1 glucosyl unit in the crystal structure of GTF180-ΔN in complex with maltose. Herein, we show that mutations in Leu^{940} of wild-type GTF180-ΔN all caused an increased percentage of (α1→6) linkages and a decreased percentage of (α1→3) linkages in the products. α-Glucans with potential different physicochemical properties (containing 67–100% of (α1→6) linkages) were produced by GTF180 and its Leu^{940} mutants. Mutant L940W was unable to form (α1→3) linkages and synthesized a smaller and linear glucan polysaccharide with only (α1→6) linkages. Docking studies revealed that the introduction of the large aromatic amino acid residue tryptophan at position 940 partially blocked the binding groove, preventing the isomalto-oligosaccharide acceptor to bind in an favorable orientation for the formation of (α1→3) linkages. Our data showed that the reaction specificity of GTF180 mutant was shifted either to increased polysaccharide synthesis (L940A, L940S, L940E, and L940F) or increased oligosaccharide synthesis (L940W). The L940W mutant is capable of producing a large amount of isomalto-oligosaccharides using released glucose from sucrose as acceptors. Thus, residue Leu^{940} in domain B is crucial for linkage and reaction specificity of GTF180. This study provides clear and novel insights into the structure-function relationships of glucansucrase enzymes.

Lactobacillus reuteri, a bacterium of human origin, has been widely used as probiotic supplement in human nutrition (1–3). *L. reuteri* strains possess glucansucrase enzymes and synthesize various α-glucans (4–8), which have potential prebiotic activities and therefore can be used to stimulate growth of beneficial intestinal bacteria such as *Bifidobacterium* and *Lactobacillus* (9). Glucansucrases are α-glucan-synthesizing enzymes only detected in lactic acid bacteria. Depending on the particular enzyme, α-glucans with different types of glycosidic linkage are produced from sucrose (10, 11): dextran with mainly (α1→6) linkages, mutan with predominantly (α1→3) linkages, reuteran containing (α1→4) and (α1→6) linkages, and alternan with alternating (α1→6) and (α1→3) linkages. A notable case is that of DSRE CD2 from *Leuconostoc mesenteroides* NRRL B-1299, forming (α1→2) single glucose branches on dextran (12). Glucansucrases catalyze three different reactions to produce glucose, polysaccharides, and oligosaccharides, using water, growing glucan chains, and low molecular mass oligosaccharides as acceptors, respectively. The structural determinants of linkage and reaction specificity (the relative balance of three reactions catalyzed) of glucansucrase are still not fully understood.

Because of their ability to produce a diverse range of α-glucans with different types of linkage, size, branching, and physicochemical properties, glucansucrases have attracted interest for industrial application such as food, medicine, cosmetics, etc. (13). Dextran produced by the glucansucrase DSRS[3] from *L. mesenteroides* NRRL B-512F was designated as a novel food ingredient by the European Union in 2001 (14). Bakery products with dextran have improved softness and increased volume (15). In addition to its use as a prebiotic food additive, α-glucan is also applied as size exclusion chromatography material in research and as a plasma expander in medicine. A more detailed understanding of the linkage and reaction specificity of glucansucrases may allow the production of tailor-made α-glucans with desired properties.

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[3] The abbreviations used are: DSR, dextransucrase; DSRS, DSR from *Leuconostoc mesenteroides* NRRL B-512F; HPAEC, high pH anion exchange chromatography.

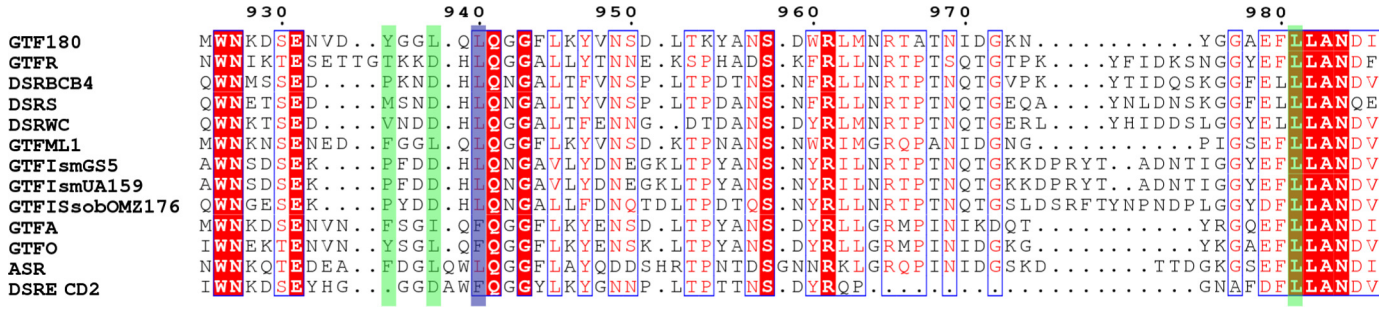

FIGURE 1. **Alignment of the glucansucrase amino acid sequences of glycoside hydrolase family 70 in the region around Leu^{940} of GTF180.** Residue Leu^{940} is highlighted in *blue*, and other residues from domain B that may be involved in product specificity of glucansucrases are highlighted in *green*.

Glucansucrases are classified in glycoside hydrolase family 70 with circularly permuted $(\beta/\alpha)_8$ barrel (16, 17). Three catalytically crucial residues of glucansucrases have been identified in previous studies (6, 17, 18). In glycoside hydrolase family 70 enzymes, Asp^{1025} (catalytic nucleophile, GTF180 numbering) is involved in the formation of a covalent glucosyl-enzyme intermediate, Glu^{1063} is the acid/base catalyst, and Asp^{1136} is the transition state stabilizer (19). Glucansucrases produce α-glucans with different structures especially regarding the glycosidic linkages. The linkage specificity of glucansucrase appears to be determined by only a small number of amino acids. In several glucansucrases, residues located C-terminally to the catalytic transition state stabilizer have been identified as important residues for linkage specificity (20–23). For example, mutations in the tripeptide (Ser^{1137}-Asn^{1138}-Ala^{1139}) following the transition state stabilizing residue (Asp^{1136}) in GTF180-ΔN of *L. reuteri* 180 altered its linkage specificity (22). Similarly, mutations in the corresponding tripeptide in GTFA-ΔN of *L. reuteri* 121 (Asn^{1134}-Asn^{1135}-Ser^{1136}) increased the amount of (α1→6) linkages and decreased the amount of (α1→4) linkages (23). Mutations P1026V and I1029V (C-terminal to the nucleophile Asp^{1024}) in GTFA-ΔN also altered linkage composition in the α-glucan produced (23). Random mutagenesis of Asp^{569} in GTF-I from *Streptococcus downei* showed that mutations at this position affected the structure of the α-glucan and the size of the synthesized oligosaccharides (24). Residues that are far away from the catalytic residues have also been found to influence the specificity of glucansucrases. For example, mutations T350K and S455K in DSRS of *L. mesenteroides* NRRL B-512F increased the amount of (α1→6) linkages in the glucan (25). Moreover, the double mutant T350K/S455K was able to produce (α1→2) branches on dextran similar to DSRE (25).

Mutants with altered linkage distribution specificity have been the main focus in previous studies. Relatively little attention has been paid to the effects of mutations on reaction specificity. Several mutant glucansucrases have been reported to produce different amounts of polysaccharides, oligosaccharides, and glucose from sucrose (20, 21, 23). For instance, S628D and S628R mutations in GTFR from *Streptococcus oralis* abolished polysaccharide synthesis and only produced short chain oligosaccharides (20). Moulis *et al.* (21) also reported that mutations in residues C-terminal to the transition state stabilizer in DSRS of *L. mesenteroides* NRRL B-512F and alternansucrase of *L. mesenteroides* NRRL B-1355 abolished or reduced polysaccharide synthesis. On the contrary, the H355V mutation in GTF-Ic of *S. downei* MFe 28 increased the production of mutan from 62 to 75% (26). Engineering of reaction specificity of glucansucrases may allow efficient production of either polysaccharides or oligosaccharides to meet the demand for different applications.

Even though various mutagenesis studies have been performed on glucansucrases, the structural features determining linkage and reaction specificity of glucansucrases are still not understood. Further studies are required to explore regions that are critical for specificity and to expand the diversity of α-glucan products for different applications. In our previous studies, GTF180 of *L. reuteri* 180 was found to produce dextran with 69% (α1→6) linkages and 31% (α1→3) linkages (5, 27). Truncation of the N-terminal variable domain had no significant effect on the product spectrum of GTF180 (5). A careful inspection of the sequence alignment of GTF180 with other glucansucrases revealed that residue Leu^{940} is highly conserved in dextran- and mutan-producing glucansucrases and in alternansucrase (Fig. 1). Instead, a phenylalanine is located at this position in reuteran-producing glucansucrases (GTFA of *L. reuteri* 121 and GTFO of *L. reuteri* ATCC 55730) and in the (α1→2)-branch forming DSRE CD2 (Fig. 1). The crystal structures of N-terminally truncated GTF180 (GTF180-ΔN) in complex with the donor substrate sucrose and the acceptor substrate maltose show that the active site is delineated by residues from domain A and B (19). Residue Leu^{940} is located in a loop of domain B, which shapes the binding groove near the acceptor binding site (19); it is close to the +1 glucosyl unit of maltose (19). The crystal structures of GTFA-ΔN (28) and DSREΔN$_{123}$-GBD-CD2 (12) showed that the corresponding phenylalanine residues (Phe^{939} and Phe^{2210}, respectively) are in the same position.

To our knowledge, residues in domain B have rarely been targeted to study their role in glucansucrase product specificity. In this study, Leu^{940} of GTF180-ΔN was chosen as a target for random mutagenesis. Our data reveal that Leu^{940} in domain B is a critical residue for glucansucrase linkage specificity, reaction specificity, and activity.

EXPERIMENTAL PROCEDURES

Sequence Alignment—Clustal Omega (29) was used to align the amino acid sequences of the dextran-producing glucansucrases GTF180 (Q5SBN3) of *L. reuteri* 180, GTFR (Q9LCH3) of *S. oralis* ATCC10557, DSRBCB4 (D2CFL0) of *L. mesenteroides* B-1299CB4, DSRS (Q9ZAR4) of *L. mesenteroides* NRRL

B-512F, and DSRWC (B9UNL6) of *Weissella cibaria* CMU; the mutan-producing glucansucrases GTFML1 (Q5SBN0) of *L. reuteri* ML1, GTFIsmGS5 (P08987) of *Streptococcus mutans* GS 5, GTFIsmUA159 (AAN58705.1) of *S. mutans* UA159, and GTFISsobOMZ176 (Q55263) of *Streptococcus sobrinus* ATCC 33478/OMZ176; the reuteran-producing glucansucrases GTFA (Q5SBL9) of *L. reuteri* 121 and GTFO (Q4JLC7) of *L. reuteri* ATCC 55730; alternansucrase ASR (Q9RE05) of *L. mesenteroides* NRRL B-1355; and the (α1→2)-forming glucansucrase DSRE CD2 (Q8G9Q2) of *L. mesenteroides* NRRL B-1299. Then the aligned sequences were submitted to EsPript (30) for alignment based on the GTF180-ΔN crystal structure (Protein Data Bank code 3KLK).

Site-directed Random Mutagenesis of Leu^{940} in GTF180-ΔN—The plasmid p15GTF180-ΔN-SX, constructed in a previous study, was used as the template for site-directed random mutagenesis (22). Random mutations of Leu^{940} were introduced by PCR with primer L940X-For (5′-CGGTGGTTTGCAANNSCAAGGTGGATTC-3′) and L940X-Rev (5′-GAATCCACCTTGSNNTTGCAAACCACCG-3′) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers were synthesized by Sigma-Aldrich. The PCR product was cleaned up with the PCR cleaning up kit (Sigma-Aldrich) and transformed into *Escherichia coli* BL21 star (DE3) (Invitrogen). After growth on LB agar plates, the colonies were inoculated in a 96-well plate with LB medium containing 100 μg/ml ampicillin. The overnight cultures were inoculated again in a new 96-well plate with fresh LB medium containing 100 μg/ml ampicillin and 0.1 mM isopropyl β-D-thiogalactopyranoside and were incubated overnight at 18 °C. Then the cells were lysed with B-PER protein extraction reagents (Thermo Scientific, Pierce) and used as crude extracts. The crude extract from each colony was incubated with 0.1 M sucrose sodium acetate buffer, pH 4.5, at 37 °C for 15 min. Glucansucrase activities were measured with 3,5-dinitrosalicylic acid reagent (31). Twenty active colonies were selected to isolate plasmid DNA. All mutations were identified by nucleotide sequencing (LGC Genomics, Berlin, Germany).

Enzyme Production and Purification—*E. coli* BL21 star (DE3) (Invitrogen) was used as a host for expression of wild-type GTF180-ΔN and all GTF180-ΔN Leu^{940} mutants. Precultures of *E. coli* BL21 star (DE3) harboring p15GTF180-ΔN-SX and different mutant plasmids were cultured overnight at 37 °C. Then fresh LB medium containing 100 μg/ml ampicillin was inoculated with 1% preculture. Expression of glucansucrases was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside when the culture reached A_{600} of 0.4–0.6. Cultivation was continued at 18 °C overnight. The cells were collected by centrifugation (10 min, 4 °C, 10,000 × *g*) and washed with 50 mM Tris-HCl buffer, pH 8.0. The (mutant) glucansucrase enzymes were purified as previously described (6).

Production of α-Glucans by Wild-type GTF180-ΔN and GTF180-ΔN Leu^{940} Mutants from Sucrose—GTF180-ΔN and mutants (1.0 unit/ml) were incubated with 0.1 M sucrose for 24 h at 37 °C in 25 mM sodium acetate buffer, 1 mM $CaCl_2$, pH 4.5. The depletion of sucrose was analyzed by TLC. The reaction was stopped by incubation at 100 °C for 10 min. The polysaccharide and oligosaccharide fractions were isolated by chromatographic separation on a Bio-Gel P-6 column (2.5 × 50 cm; Bio-Rad) using 10 mM NH_4HCO_3 as eluent at a flow rate of 48 ml/h. Free fructose was not collected.

High pH Anion Exchange Chromatography with Pulsed Amperometric Detection Analysis—The oligosaccharides produced by GTF180-ΔN (mutants) were analyzed by high pH anion exchange chromatography (HPAEC) on a Dionex DX500 work station, equipped with an ED40 pulsed amperometric detection system. The oligosaccharides were separated on a CarboPac PA-1 column (250 × 5 mm; Dionex) by using a linear gradient of 10–240 mM sodium acetate in 100 mM NaOH (1 ml/min).

^{1}H NMR Spectroscopy—^{1}H NMR spectra of polysaccharides and oligosaccharides produced by different mutants were recorded on a Varian Inova 500 Spectrometer (NMR Center, University of Groningen) at probe temperatures of 300 K. Samples were exchanged twice with 99.9%$_{atom}$ D_2O (Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization and dissolved in 600 μl of D_2O. Chemical shifts were expressed in ppm and calibrated by internal standard acetone (δ 2.225). The percentage of different linkages was estimated by integration of the respective signal peak areas.

Determination of the Reaction Specificity—The reaction specificity was defined as the relative balance of the three reactions (hydrolysis, oligosaccharide synthesis, and polysaccharide synthesis) catalyzed by glucansucrases. Changes in reaction specificity were monitored by determining the percentage of sucrose used for polysaccharide synthesis, oligosaccharide synthesis, and hydrolysis. The percentages of sucrose used for hydrolysis and polysaccharide synthesis were determined by detecting the released glucose in the incubation mixture and the amount of glucose in the polysaccharide, respectively. The percentage of sucrose used for oligosaccharide synthesis was calculated by subtracting the amount of sucrose for hydrolysis and polysaccharide synthesis from the total amount of sucrose added to the incubation mixture. The amount of glucose released in the incubation mixture was measured by converting the glucose into gluconate-6-phosphate with hexokinase and glucose-6-phosphate dehydrogenase and measuring the NADH release at A_{340} (32). The amount of glucose in the isolated polysaccharide was determined by measuring the weight of polysaccharides after lyophilization. The amounts of polysaccharide synthesis, oligosaccharide synthesis, and hydrolysis were expressed as the percentages of sucrose used for each reaction.

Enzymatic Activity Assays—The enzymatic activities of GTF180-ΔN (mutants) were measured as previously described (6). Briefly, enzyme assays were performed with 30 nM enzyme in 25 mM sodium acetate buffer, pH 4.5, at 50 °C. Samples of 25 μl of incubation mixture were withdrawn every 1 min for 5 min and inactivated with 2.5 μl of 1 M NaOH. One unit of enzyme activity was defined as the release of 1 μmol of fructose per min. The kinetic parameters (K_m and k_{cat}) were determined with 12 different sucrose concentrations ranging from 0.5 to 200 mM. The kinetic parameters were calculated using SigmaPlot version 12.5.

Oligosaccharide Synthesis with 0.1 M Maltose as Acceptor by GTF180-ΔN Leu^{940} Mutants from Sucrose—GTF180-ΔN and mutants (1.0 unit/ml) were incubated with 0.1 M sucrose and 0.1

TABLE 1
Structural properties of polysaccharides and oligosaccharides produced by wild-type GTF180-ΔN and Leu^{940} mutants

Enzymes	Polysaccharide chemical shift[a]		Molecular mass[b]	Relative molecular mass	Oligosaccharide chemical shift[a]	
	(α1→6)	(α1→3)			(α1→6)	(α1→3)
	%		1×10^6 Da	%	%	
GTF180-ΔN	67	33	22.6	100.0	78	22
L940G	85	15	16.8	74.1	92	8
L940C	74	26	17.3	76.3	85	15
L940A	84	16	19.3	85.2	91	9
L940S	84	16	19.7	87.0	91	9
L940M	72	28	19.2	84.7	80	20
L940E	73	27	18.7	82.5	83	17
L940F	93	7	19.5	86.3	95	5
L940W	100	0	6.3	27.9	100	0

[a] The data represent the ratios of integration of the surface areas of the (α1→6) linkage signal at 4.96 ppm and the (α1→3) linkage signal at 5.33 ppm in the ^{1}H NMR spectra of the polysaccharides and oligosaccharides produced.
[b] The average molecular mass of polysaccharides was determined in duplicate.

M maltose for 24 h at 37 °C. The reaction was stopped by incubation at 100 °C for 10 min. The amounts of remaining maltose and panose produced in the incubation mixture were determined by HPAEC pulsed amperometric detection with the respective standards. Because of the lack of a standard for glucosyl-(α1→6)-panose (α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*) and [glucosyl-(α1→6)]$_2$-panose (α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*), their concentrations were estimated using panose as the standard. Different oligosaccharide yields were calculated as the percentages of the amount of maltose converted to the respective oligosaccharides. Approximately 90–100% of maltose initially present in the incubation was recovered from the analysis. The lower percentages may be due to the use of the D-panose calibration curve to determine the amount of glucosyl-(α1→6)-panose and [glucosyl-(α1→6)]$_2$-panose.

Size Determination of Polysaccharide Produced by GTF180-ΔN Leu^{940} Mutants—The size of polysaccharides was determined using a SEC system (Agilent Technologies 1260 Infinity) from Polymer Standards Service (Mainz, Germany). The setup consisted of an isocratic pump, an auto sampler without temperature regulation, an online degasser, an inline 0.1-μm filter, a refractive index detector (G1362A 1260 RID; Agilent Technologies), and multiangle laser light scattering (SLD 7000; Polymer Standards Service). As eluent, 0.1 M Na_2SO_4 with 0.02% NaN_3 was used at a flow rate of 0.8 ml/min. Polysaccharide samples were also dissolved in 0.1 M Na_2SO_4 with 0.02% NaN_3. The samples (100 μl) were injected into a Suprema guard column and three Suprema SEC columns: 100, 3000, and 30000. Columns and detectors were kept at 50 °C. A standard pullulan kit (Polymer Standards Service) with molecular masses from 342 to 805,000 Da was used for making a calibration curve. The multiangle laser light scattering signal was used to assess the molar masses with a refractive index increment value (*dn*/*dc*) of 0.147 ml/g. WinGPC Unity software (Polymer Standards Service) was used for data processing. Measurements were performed in duplicate.

Docking Studies—Docking studies of wild-type GTF180-ΔN and GTF180-ΔN L940W were performed with isomaltotriose, essentially as described previously (19). Briefly, all the dockings were carried out using an energy-minimized model of the glucosyl-enzyme covalent intermediate of GTF180-ΔN. Hydrogen atoms and charges were added using the AMBER (33) force field, and the docking space was chosen such that it included subsite +1 as well as the space near residue 940. The effects of the L940W mutation were evaluated using two top-ranked side chain rotamers according to the Dunbrack rotamer library (34). For each case (wild-type and two L940W rotamers), 1000 poses of isomaltotriose were calculated using Autodock 4 (35). Inverted configurations (*i.e.* with the reducing and nonreducing ends of the trisaccharide switched) were not considered in the study.

RESULTS AND DISCUSSION

Construction and Expression of GTF180-ΔN Leu^{940} Mutants—The mutations were introduced by PCR using appropriate primers. The PCR library containing random mutations was transformed into *E. coli* BL21 DE3 star. Twenty clones were selected based on activity as described under "Experimental Procedures." Sequencing of 20 clones revealed five of L940G, one of L940C, two of L940A, two of L940S, two of L940M, one of L940E, four of L940F, and one of L940W. Thus, eight different GTF180-ΔN Leu^{940} mutants were found in total. Wild-type GTF180-ΔN and the eight Leu^{940} mutants were expressed and purified. Compared with GTF180-ΔN, no significant difference in expression levels of the mutants was observed. The eight GTF180-ΔN Leu^{940} mutants covered various classes of amino acids with the exception of amino acids with a positive charge.

Effects of Mutation on Linkage Specificity and Size of α-Glucan Polysaccharides Produced from Sucrose—To explore the effects of mutations at position 940 on the linkage specificity of GTF180-ΔN, the α-glucans produced were analyzed by ^{1}H NMR spectroscopy. The results showed that all eight mutants synthesized α-glucans with a higher percentage of (α1→6) linkages than that of GTF180-ΔN (Table 1). Based on the extent of the shift to (α1→6) linkages, three different groups of mutants were observed. First, mutations of Leu^{940} to similar size amino acids (cysteine, methionine, and glutamate) showed a relatively small shift with ~73% of (α1→6) linkages in the polysaccharide produced (Table 1). A larger shift was observed when the mutant residue (L940G, L940A, and L940S) has a smaller side chain than the wild-type leucine. Mutants L940G, L940A, and L940S produced α-glucans with similar percentages of (α1→6) linkages, which shifted from 67% in wild-type GTF180-ΔN to 85% (Table 1). The largest shift was observed when a large aromatic side chain was introduced (L940F and L940W). Mutation

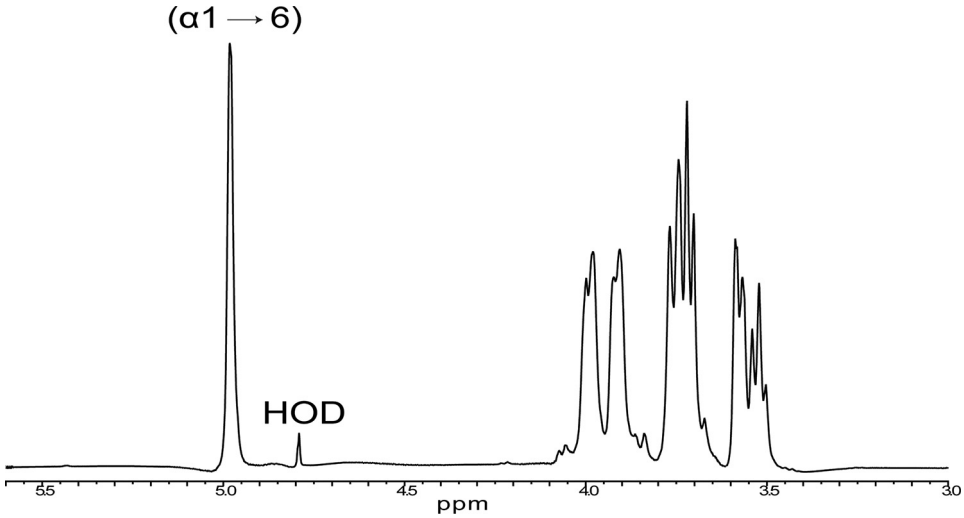

FIGURE 2. **The one-dimensional ^{1}H NMR spectrum of α-glucan produced by GTF180-ΔN L940W mutant recorded at 300 K in D_2O.**

of Leu^{940} to the bulky residue phenylalanine, which is present in reuteransucrase GTFA and GTFO, creating different ratios of (α1→4) and (α1→6) linkages (4, 6), and DSRE CD2, creating (α1→2) branch linkages on dextran (12), caused a shift in the percentage of (α1→6) linkages to 93% (Table 1). However, the L940F mutation did not enable the synthesis of (α1→4) or (α1→2) linkages. Thus, other residues must be involved in determining the synthesis of (α1→4) and (α1→2) linkages. Notably, mutation of Leu^{940} to tryptophan abolished (α1→3) linkage synthesis completely, resulting in synthesis of α-glucans with (α1→6) linkages exclusively (Fig. 2). Typically, glucansucrases catalyze the synthesis of α-glucans with two types of linkage (10). To our knowledge, our present study is the first to report that a single mutation completely abolished one type of linkage in the synthesis of α-glucan by a glucansucrase. The polysaccharide produced by wild-type GTF180-ΔN is built up from different lengths of isomalto-oligosaccharides, interconnected by single (α1→3) glycosidic linkages in linear and branched orientations (12% terminal, 24% 3-monosubstituted, 52% 6-monosubstituted, and 12% 3,6-disubstituted α-D-glucopyranose residues) (27). The L940W mutant is deficient in the synthesis of (α1→3) branch linkages, resulting in the synthesis of linear α-glucans. Until now, it has not been understood how product specificity (including linkage specificity) is determined in glucansucrases; it appears to involve an interplay of residues surrounding the active site that affect the binding mode and orientation of acceptor sugars. In particular, residues from the catalytic domain (domain A), especially those in the region following the transition state stabilizing residue, seem to play an important role (20–24). In our present study, the linkage distribution of the α-glucans produced by Leu^{940} mutants clearly suggests that Leu^{940} of domain B plays an important role in linkage specificity of GTF180-ΔN. Although residue Leu^{940} in GTF180-ΔN is not close to the catalytic residues (the distance to Asp^{1025}/Glu^{1063} is ~10–11 Å), its side chain points toward the substrate/acceptor binding groove and is located at 6–8 Å distance from the +1 glucosyl moiety of the bound maltose (Protein Data Bank code 3KLL (19)). The clear influence of the size of the (mutant) side chain at position 940 on linkage specificity suggests that steric effects may affect acceptor binding and thus determine which linkage type is favored. In a recent combinatorial engineering study of DSRS from *L. mesenteroides* NRRL B-512F, residue Phe^{353} (DSRS numbering, corresponding to Ala^{978} in GTF180) of domain B was shown to slightly alter linkage specificity (36). The present study indicates that not only residues from domain A but also from domain B of glucansucrase are critical for linkage specificity determination.

Regarding product size, the polysaccharides produced by mutants L940A, L940S, L940M, L940E, and L940F were similar to that of wild-type GTF180-ΔN (Table 1). L940G and L940C synthesized ~25% smaller polysaccharides than that of GTF180-ΔN (Table 1). Notably, the α-glucan polysaccharide synthesized by mutant L940W showed a size reduction of 70%. Previous studies have reported glucansucrase mutations that resulted in synthesis of polysaccharides with reduced molecular masses (22, 37, 38), but a clear picture of the mechanism of size determination has not emerged. Mutations at position 940, lining the binding groove, may affect the affinity for acceptor molecules during product elongation and thus the final size of the polysaccharides.

Effects of Mutations on Oligosaccharide Synthesis from Sucrose Only and from Sucrose and Maltose—The altered linkage specificity of GTF180-ΔN Leu^{940} enzyme variants was also observed in the oligosaccharides produced from sucrose (Table 1). For each enzyme, the percentage of (α1→6) linkages in the oligosaccharides is higher than that in the polysaccharides produced (Table 1). There is a significant positive correlation between the number of (α1→6) linkages in the polysaccharide and the amount of (α1→6) linkages in the oligosaccharides. It is noteworthy that the HPAEC (CarboPac PA-1) profile of the oligosaccharides produced by the L940W mutant showed a relatively simple pattern of peaks with increasing degrees of polymerization (according to MALDI-TOF-MS) (Fig. 3*A*). The peaks were assigned by combining NMR data of the oligosaccharide pool and by comparison of the retention times with those of reference compounds (Fig. 3). The ^{1}H NMR spectrum of the oligosaccharide pool produced by the L940W mutant

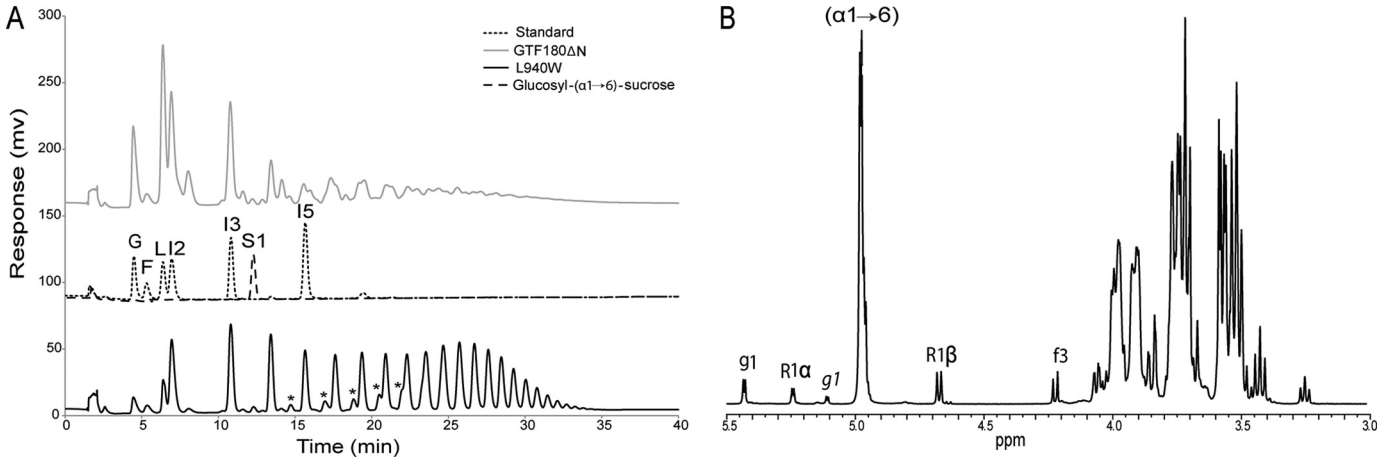

FIGURE 3. *A*, HPAEC (CarboPac PA-1) analysis of the oligosaccharides synthesized by the wild-type GTF180-ΔN and L940W mutant enzymes using 100 mM sucrose as substrate. The *y* axis scales of chromatograms were elevated to better visualize oligosaccharide chromatograms. *G*, glucose; *F*, fructose; *L*, leucrose; *I2*, isomaltose; *I3*, isomaltotriose; *I5*, isomaltopentaose; *S1*, glucosyl-(α1→6)-sucrose. *, minor amounts of oligosaccharides that are expected to stem from glucosyl-(α1→6) elongations of S1 and coelute with the isomalto-oligosaccharides at higher degrees of polymerization. *B*, the one-dimensional ^{1}H NMR spectrum of the oligosaccharide fraction obtained after incubation of sucrose with the L940W mutant enzyme.

enzyme with sucrose as substrate (Fig. 3*B*) showed one major signal and four minor signals in the anomeric region (δ 4.5–5.5). The predominant signal at δ 4.96 belongs to successive (α1→6) linkages (39). The ^{1}H chemical shifts of the minor anomeric signals at δ 5.242 (R1α) and 4.675 (R1β) indicate the presence of 6-substituted reducing Glc*p* residues (39). Typical chemical shift values for sucrose fragments (Glc, g; Fru, f) are δ 5.431, stemming from Glc g H-1, and δ 4.223, stemming from Fru f H-3 (39). The anomeric signal at δ 5.110 (Glc, g) indicates the presence of leucrose (α-D-Glc*p*-(1→5)-β-D-Fru*f*) (40). The NMR data showed that released glucose and sucrose were used as acceptors by the L940W mutant and that they are further elongated with successive (α1→6) linkages. Fructose was also used as acceptor to produce leucrose. The majority of the oligosaccharide products produced by the L940W mutant from sucrose are linear isomalto-oligosaccharides with increasing degrees of polymerization, whereas sucrose-containing oligosaccharides and leucrose are present in lesser amounts (Fig. 3*B*). Therefore, the L940W mutant is efficient in the synthesis of isomalto-oligosaccharides from sucrose and holds great potential for the production of isomalto-oligosaccharides. However, the HPAEC profiles of oligosaccharides produced by the wild-type GTF180-ΔN enzyme (Fig. 3*A*) and other mutants (data not shown) showed very complex oligosaccharide mixtures. The wild-type GTF180-ΔN and other mutants produced oligosaccharides containing both (α1→6) and (α1→3) linkages using glucose and sucrose as acceptors (Table 1). Here, fructose was also used as acceptor to produce leucrose.

With the availability of maltose as acceptor, GTF180-ΔN catalyzes the synthesis of panose, glucosyl-(α1→6)-panose (α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*), and [glucosyl-(α1→6)]$_2$-panose (α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*), etc. (19). Table 2 shows that there are no significant differences in the amounts of panose and glucosyl-(α1→6)-panose produced by wild-type GTF180-ΔN and Leu^{940} mutants in the presence of 100 mM sucrose (donor) and 100 mM maltose (acceptor). This result may be explained by the fact that maltose (as an acceptor) binds

TABLE 2

Oligosaccharides produced by wild-type GTF180-ΔN and its Leu^{940} mutants in the presence of 100 mM maltose and 100 mM sucrose

Enzymes	Remaining maltose[a]	Panose	Glucosyl-(α1→6)-panose[b]	[Glucosyl-(α1→6)]$_2$-panose[b]
GTF180-ΔN	45.7 ± 3.8	29.4 ± 1.2	14.8 ± 0.9	2.7 ± 0.2
L940G	44.3 ± 3.2	31.3 ± 2.3	18.1 ± 2.6	6.5 ± 0.8
L940C	41.5 ± 4.5	29.6 ± 2.2	15.9 ± 1.8	3.7 ± 0.2
L940A	42.7 ± 4.2	28.8 ± 3.0	16.5 ± 1.2	4.3 ± 0.3
L940S	41.9 ± 3.1	28.6 ± 1.9	16.8 ± 0.7	4.4 ± 0.2
L940M	45.7 ± 2.0	36.9 ± 4.0	15.0 ± 0.6	2.3 ± 0.3
L940E	44.3 ± 4.3	27.8 ± 1.8	14.0 ± 0.9	3.4 ± 0.1
L940F	43.2 ± 4.4	24.1 ± 3.6	17.6 ± 2.1	8.0 ± 0.4
L940W	44.8 ± 2.8	22.6 ± 1.8	15.4 ± 1.4	8.9 ± 0.6

[a] The remaining maltose and individual oligosaccharides were quantified and expressed as the percentages of maltose consumed of the total amount of maltose initially present in the incubations.

[b] The calibration curve of panose was used to quantify the amount of glucosyl-(α1→6)-panose (α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*) and [glucosyl-(α1→6)]$_2$-panose (α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*) due to lack of standards.

"away" from the position of Leu^{940} (19). In other words, the mutations at position Leu^{940} are not close enough to the maltose binding site to affect the linkage specificity of oligosaccharides produced. However, a positive correlation was found between the percentage of (α1→6) linkages in the polysaccharides and the amount of [glucosyl-(α1→6)]$_2$-panose produced by GTF180-ΔN and Leu^{940} mutants in the presence of 100 mM sucrose and 100 mM maltose (Table 2). Thus, the altered linkage specificity was also reflected in the oligosaccharide synthesis from sucrose and maltose. Monchois *et al.* (24) also reported that no additional new oligosaccharides were produced by Asp^{569} mutants of GTF-I from *S. downei* in the presence of maltose and sucrose. However, the distribution of different sizes of oligosaccharides was affected by using different concentrations of maltose and sucrose (24).

Effects of Mutation on Reaction Specificity—Several of the mutations at residue 940 in GTF180-ΔN affected the reaction specificity, reflecting in the relative amounts of sucrose used for hydrolysis, oligosaccharide synthesis, and polysaccharide synthesis, respectively (Table 3). Wild-type GTF180-ΔN used 23.9,

TABLE 3
Effects of mutations at residue Leu^{940} of GTF180-ΔN on the reaction specificity

Enzymes	Hydrolysis[a]	Polysaccharide synthesis	Oligosaccharide synthesis
GTF180-ΔN	23.9 ± 0.7	16.3 ± 0.7	59.7 ± 1.2
L940G	30.1 ± 0.3	8.9 ± 0.2	61.0 ± 0.4
L940C	32.0 ± 0.7	8.1 ± 0.3	59.9 ± 0.9
L940A	26.3 ± 1.0	22.3 ± 0.4	51.4 ± 0.7
L940S	27.9 ± 0.3	21.4 ± 0.2	50.7 ± 0.2
L940M	24.4 ± 0.9	18.6 ± 0.1	57.0 ± 0.9
L940E	23.9 ± 0.8	29.9 ± 0.3	46.2 ± 0.9
L940F	15.8 ± 0.1	30.4 ± 0.5	53.8 ± 0.5
L940W	3.9 ± 0.4	4.3 ± 0.1	91.8 ± 0.5

[a] The values show the percentages of sucrose used for hydrolysis, polysaccharide synthesis, and oligosaccharide synthesis of the total amount of sucrose present initially in the incubations.

TABLE 4
Kinetic properties of wild-type GTF180-ΔN and Leu^{940} mutants

Enzyme[a]	K_m	k_{cat}	k_{cat}/K_m
	mM	s^{-1}	s^{-1} mM^{-1}
GTF180-ΔN	5.0 ± 0.3	303.0 ± 3.6	60.6
L940G	9.0 ± 1.0	134.0 ± 3.1	14.9
L940C	24.1 ± 1.5	253.8 ± 4.8	10.5
L940A	27.9 ± 2.1	135.6 ± 4.9	4.9
L940S	20.1 ± 2.6	84.0 ± 4.0	4.2
L940M	7.9 ± 0.5	237.0 ± 3.2	30
L940E	19.3 ± 2.6	117.9 ± 4.0	6.1
L940F	7.1 ± 0.4	158.7 ± 1.8	22.4
L940W	21.1 ± 2.2	190.7 ± 6.1	9.0

[a] The kinetic parameters (K_m and k_{cat}) were determined with 12 different sucrose concentrations ranging from 0.5 to 200 mM.

16.3, and 59.7% of sucrose for hydrolysis, polysaccharide synthesis and oligosaccharide synthesis, respectively (Table 3). The hydrolysis of L940G and L940C accounted for more than 30% of sucrose added, whereas the percentage for polysaccharide synthesis decreased to ~8% (Table 3). As seen for linkage specificity, mutants L940A and L940S also behaved similarly regarding reaction specificity with only a slight increase in hydrolysis and an increased sucrose consumption for polysaccharide synthesis (~22%; Table 3). The L940M mutation had no effect on reaction specificity (Table 3), which may be explained by the similar properties of leucine and methionine (both are hydrophobic and similar in size). The L940E and L940F mutants both showed a significant increase in polysaccharide synthesis (to ~30% of sucrose). Hydrolysis of L940E remained the same as in GTF180-ΔN, whereas in L940F, hydrolysis was reduced to 16%. The strongest reduction in hydrolysis was observed with L940W: it decreased 6-fold compared with wild-type GTF180-ΔN. A possible explanation is that the tryptophan side chain increases the hydrophobicity of the active site and/or partially shields off a bound sucrose for attack by water. Reducing hydrolysis is an important aspect in engineering of glucansucrase enzymes; the L940W mutation thus provides a promising starting point for further engineering. In addition, the percentage of sucrose utilization for polysaccharide synthesis of L940W was reduced to ~4.3%, whereas oligosaccharide synthesis increased to 91.8%.

Overall, our results show that mutations of Leu^{940} in GTF180-ΔN affect reaction specificity significantly. Most mutation studies involving glucansucrases have focused on changing linkage specificity (10, 11, 41), whereas only a few have investigated the effects of mutations on reaction specificity. For example, mutations S628D and S628R in GTFR from *S. oralis* were reported to have a heavily impaired polysaccharide synthesis and synthesize many more short chain oligosaccharides instead (20). Mutations at Leu^{940} in GTF180-ΔN showed that the relative balance of the three reactions could be engineered toward either polysaccharide synthesis (L940A, L940S, L940E, and L940F) or oligosaccharide synthesis (L940W).

Effects of Mutation on Kinetic Parameters of Enzymes—The kinetic parameters (K_m and k_{cat}) determined for wild-type GTF180-ΔN and Leu^{940} mutants are summarized in Table 4. In general, all Leu^{940} mutants showed an increased K_m for sucrose. Compared with wild-type GTF180-ΔN, the K_m values of L940G, L940M, and L940F showed only a minor increase, whereas L940C, L940A, L940S, L940E, and L940W showed a 4–5-fold increase in K_m. L940C and L940M showed only slightly decreased k_{cat} values compared with that of wild-type GTF180-ΔN, whereas the k_{cat} values of the other mutants were reduced approximately by 2–4-fold (Table 4). Again, L940A and L940S behaved similarly, as previously shown for linkage and reaction specificity (Tables 1, 3, and 4). Mutation to methionine had the smallest effects (only 50% reduction) on activity (k_{cat}/K_m), possibly because of its similar properties with leucine. Taken together, all mutants showed a decrease in catalytic efficiency (k_{cat}/K_m; Table 4), which may be due to the fact that mutations change the shape of the binding groove and thus may affect the affinity (19, 41).

Docking Studies—Linkage type distribution analysis showed that, using sucrose, mutant L940W is incapable of (α1→3) linkage formation, and only catalyzes the synthesis of (α1→6) linkages (Table 1). Another interesting feature of the L940W mutant is its 6-fold decreased hydrolysis. The available high resolution crystal structure of GTF180-ΔN and a model of its covalent glucosyl-enzyme intermediate (19) allowed us to investigate the structural effects of the L940W mutation, by performing docking experiments using isomaltotriose (α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glc*p*) as the ligand. The top-ranked mutants are shown in Fig. 4; the results are consistent with those from product analysis and kinetic analysis described above.

Previously, it was shown that in wild-type GTF180-ΔN, docked (α1→6)-linked gluco-oligosaccharides adopt conformations favorable for either (α1→6)- or (α1→3)-linkage formation, although the former is preferred (19). In our docking experiments with wild-type GTF180-ΔN, isomaltotriose indeed adopted a conformation where the O3 hydroxyl group of the nonreducing end glucosyl unit closely interacts with the C1 atom of the covalent glucosyl-enzyme intermediate to form an (α1→3) linkage (this conformation was similar to the previously reported one, except that the trisaccharide is shifted by one glucosyl unit because of the definition of the docking space box). The other two glucosyl units (subsites +2 and +3) were located near residue Leu^{940} (Fig. 4*A*), in the groove lined on the one side by residues of domain B.

Docking experiments with the GTF180-ΔN L940W mutant revealed important differences regarding the configurational space for isomaltotriose. When applying the most energetically stable rotamer of L940W (the first rotamer; Fig. 4*B*), the tryptophan side chain was observed to block the

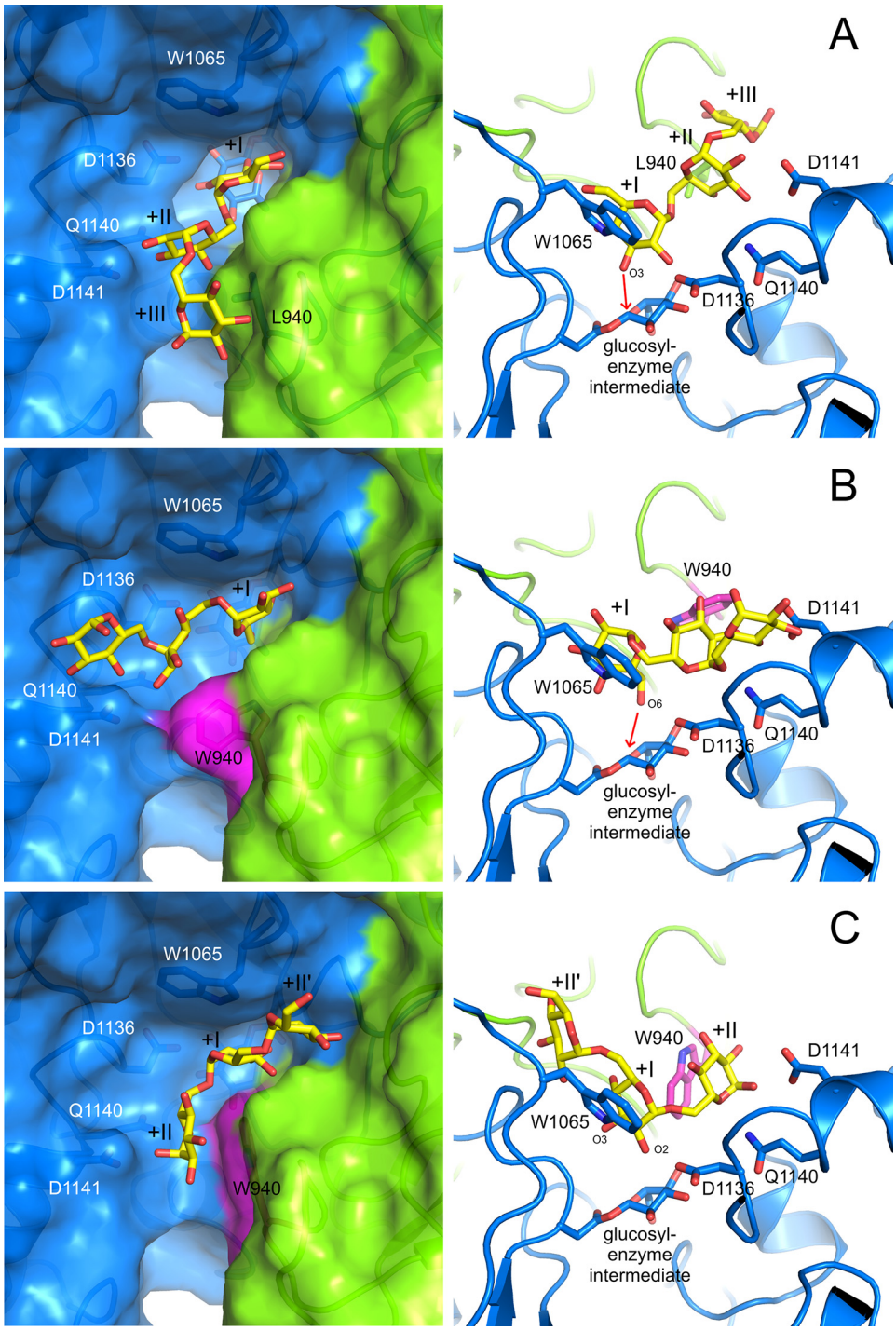

FIGURE 4. **Molecular docking of isomaltotriose in the acceptor binding site of GTF180-ΔN.** Residues of domain A are depicted in *blue*; residues of domain B are in *green*. *A*, wild-type GTF180-ΔN. The O3 of the glucosyl unit bound in subsite +1 is at close distance (3.1 Å, see *arrow*) from the C1 atom of the covalent glucosyl-enzyme intermediate. *B*, in mutant L940W rotamer 1, the tryptophan side chain blocks the groove and forces the reducing end of the trisaccharide toward residues from the loop following residue Asp^{1136}, the transition state stabilizing residue. Consequently, the glucosyl unit in subsite +1 cannot orient its C3-hydroxyl toward the glucosyl-enzyme intermediate; rather its C6-hydroxyl group is at closer distance to form an (α1→6) linkage. *C*, in rotamer 2 of Trp^{940}, the shape of the groove is less affected. Nevertheless, the trisaccharide is reoriented such that at subsite +1 neither of the hydroxyl groups is close enough to form a glycosidic linkage.

groove entrance, preventing the reducing end units of isomaltotriose to occupy the space observed in the wild-type enzyme. Instead, the ligand reoriented in such a way that the reducing end sugar moiety was now bound near residues Asn^{1138}, Asp^{1141}, and Gln^{1142}. Importantly, the C3 hydroxyl group of the glucosyl unit bound in subsite +1 is located too far from the C1 atom of the glucosyl-enzyme intermediate. Instead, the C6 hydroxyl group is now found to be well positioned for the formation of an (α1→6) linkage. In the case of the second rotamer of L940W, the tryptophan side chain aligned with the groove surface (Fig. 4*C*), modifying the shape of the groove to a lesser extent than that of the first

rotamer. Although isomaltotriose was still able to sample similar configurations as observed in the wild-type enzyme, the orientation of the middle glucosyl unit did not allow the C3 hydroxyl group of the middle glucosyl unit to approach the C1 atom of the glucosyl-enzyme intermediate close enough to favor glycosidic linkage formation.

In summary, the docking studies showed that the binding mode of isomaltotriose is constrained by the groove shape that is lined (among others) by residue Leu^{940}, which is positioned near subsites +2 and +3 of this trisaccharide (Fig. 4*A*). Introduction of the bulky tryptophan side chain forces the trisaccharide to adopt a different conformation, such that the subsite +1 glucosyl no longer favors (α1→3) linkage formation, while (α1→6) linkage formation is retained. Thus, these results explain the altered linkage specificity of the L940W mutant and indicate the importance of steric effects for acceptor binding. Indeed, a structural inspection of other mutations (Gly, Cys, Ala, Ser, Met, Glu, or Phe) at position 940 showed that they all change the shape of the binding groove (data not shown), apparently disfavoring (α1→3) linkage formation.

Conclusions—Our data show that residue 940 of GTF180-ΔN is of critical importance for linkage specificity, reaction specificity, and activity. Mutation of Leu^{940} to eight different residues (Gly, Cys, Ala, Ser, Met, Glu, Phe, and Trp) all caused a shift in linkage specificity to (α1→6) linkage in the polysaccharides and oligosaccharides synthesized from sucrose only or from both sucrose and maltose. Interestingly, in mutant L940W, (α1→3) linkage synthesis was completely abolished, and a smaller linear polysaccharide with only (α1→6) linkages was synthesized. Docking studies provide molecular insight into how the introduction of the tryptophan side chain prevents (α1→3) linkage-favoring acceptor orientation and point to the importance of steric effects for linkage specificity.

In addition to the altered linkage specificity, our data also showed that the reaction specificity can be engineered to increase either polysaccharide synthesis (L940A, L940S, L940E, and L940F) or oligosaccharide synthesis (L940W) or to reduce hydrolysis (L940W). The L940W mutant is capable of producing a large amount of isomalto-oligosaccharides from sucrose. All Leu^{940} mutants retained a relatively high activity even though their K_m was increased and k_{cat} was reduced.

α-Glucans containing 67–100% of (α1→6) linkages are produced by GTF180-ΔN and Leu^{940} mutants and may show different physicochemical properties. Given the conservation of residue Leu^{940} and its equivalents among glucansucrases (only leucine or phenylalanine is observed), the importance of this residue for product specificity likely extends to other glucansucrases. This may also be true for other residues from domain B, such as Tyr^{935}, Leu^{938}, and Leu^{981} in GTF180-ΔN (Fig. 1), shaping the binding groove at the same side. Supplementing previously reported residues from domain A, these residues provide new targets for enzyme engineering, aiming at tailor-made products with novel properties. Our study provides novel insights into the structure-function relationships of glucansucrases regarding their linkage specificity, reaction specificity, and activity.

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